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Intragenomic heterogeneity in the 16S rRNA genes of *Flavobacterium columnare* and standard protocol for genomovar assignment

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Abstract

Genetic variability in 16S rRNA gene sequences has been demonstrated among isolates of *Flavobacterium columnare,* and a restriction fragment length polymorphism (RFLP) assay is available for genetic typing of this important fish pathogen. Interpretation of restriction patterns can be difficult due to the lack of a formal description of the expected number and sizes of DNA fragments generated for each of the described genomovars. In this study, partial 16S rRNA gene sequences (ca. 1250-bp fragment) from isolates representing each described genomovar and isolates generating unique restriction patterns were cloned and sequenced. The results demonstrated that some isolates contained up to three different 16S rRNA genes whose sequences generate different RFLP patterns due to intragenomic heterogeneity within *Hae*III restriction sites. The occurrence of *Hae*III restriction sites within the portion of the 16S rRNA gene used for typing the *F. columnare* isolates and intragenomic heterogeneity within these sites explained the restriction patterns observed following RFLP analyses. This research provides a standard protocol for typing isolates of *F. columnare* by RFLP and a formal description of the expected restriction patterns for the previously described genomovars I, II, II-B and III. Additionally, we describe a new genomovar, I/II.

Keywords: 16S rRNA, columnaris disease, *Flavobacterium columnare*, genomovar, intragenomic heterogeneity, restriction fragment length polymorphism.

Introduction

Flavobacterium columnare is a Gram-negative bacterial pathogen of fish that causes columnaris disease and contributes to substantial losses in worldwide aquaculture production. In the USA, columnaris disease negatively impacts the channel catfish *Ictalurus punctatus* (Rafinesque) industry (Wagner *et al.* 2002) and may be emerging as a significant disease of rainbow trout *Oncorhynchus mykiss* (Walbaum; LaFrentz *et al.* 2012).

Triyanto & Wakabayashi (1999) demonstrated genetic variability among *F. columnare* isolates and developed a 16S rRNA gene–based restriction fragment length polymorphism (RFLP) assay. Using this assay, three distinct genetic groups were identified and described as genomovars I, II and III. Additional research utilizing this assay suggested that subgroups within genomovars I and II existed, which were classified as genomovar I-B and genomovar II-B (Olivares-Fuster *et al.* 2007b). The establishment of this genetic typing system prompted research to determine whether there are associations between genomovar and virulence or host of origin. Shoemaker *et al.* (2008)

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demonstrated that genomovar II isolates were more virulent in channel catfish than genomovar I isolates, and similar findings were suggested in zebrafish *Danio rerio* (Hamilton; Olivares-Fuster *et al.* 2011). Recently, LaFrentz *et al.* (2012) demonstrated that genomovar II isolates were more virulent than genomovar I isolates using rainbow trout as a model, providing further support for an association between genomovar and virulence. Research has also suggested host-specific associations between genomovars and fish species. Olivares-Fuster *et al.* (2007a) demonstrated a host-specific association between genomovar I isolates and threadfin shad *Dorosoma petenense* (Günther) following isolation of *F. columnare* from wild fish in the Mobile River (Alabama, USA). LaFrentz *et al.* (2012) suggested that there may be a similar association between genomovar I isolates and salmonids based on the observation that all *F. columnare* isolates from salmonids that had been typed and published belonged to genomovar I. The aforementioned findings suggest that the RFLP typing system for *F. columnare* is useful because genetic type appears to have biological relevance.

Although the RFLP method for *F. columnare* has been used by different laboratories (Triyanto & Wakabayashi 1999; Michel, Messiaen & Bernardet 2002; Arias *et al.* 2004; Darwish & Ismaiel 2005; Schneck & Caslake 2006; Suomalainen *et al.* 2006; Avendaño-Herrera *et al.* 2011; LaFrentz *et al.* 2012), there has been no formal description of the expected restriction patterns for each genomovar. This lack of knowledge can make results difficult to interpret and requires comparison of observed restriction patterns to published gel images. The primary difficulty in interpretation is variability between studies in the number and sizes of DNA fragments generated following *Hae*III digestion of amplified DNA. Such variability may be explained by the use of different methods and/or reagents, such as types of agarose, electrophoresis buffers/conditions and PCR primers. This becomes problematic when assigning an unknown *F. columnare* isolate to a genomovar, especially when the observed restriction pattern is unique or differs slightly from published patterns. This became apparent as our laboratory began typing new *F. columnare* isolates recovered from fish exhibiting columnaris disease and upon inclusion of archived isolates for typing controls. During initial efforts to type these isolates, we began to critically evaluate our observed restriction patterns comparing them with those that had been previously published. These efforts were met with difficulty due to the aforementioned reasons. Additionally, for some isolates, the cumulative molecular weight (bp) of all DNA fragments generated following digestion with *Hae*III was greater than the size of the PCR product originally used for digestion. We hypothesized that there was nucleotide variability or intragenomic heterogeneity among multiple copies of the 16S rRNA gene in these isolates and the heterogeneity occurred within *Hae*III restriction sites. This would result in multiple copies of the 16S rRNA gene producing different restriction patterns, which could account for the discrepancy between the cumulative molecular weight of all DNA fragments and the undigested PCR product. Many bacterial species have multiple copies of the 16S rRNA gene encoded in their genome (Pei *et al.* 2010), including *F. columnare* (Tekedar *et al.* 2012), and there is a precedent for intragenomic heterogeneity among these (Coenye & Vandamme 2003).

Therefore, we pursued research to formally describe the expected restriction patterns for each genomovar as well as isolates generating unique restriction patterns. The specific objectives of this study were to (i) determine optimal electrophoresis conditions for increased resolution of DNA fragments following digestion, (ii) clone and sequence the portion of the 16S rRNA gene used for RFLP from isolates representing different genomovars or generating unique restriction patterns to determine whether intragenomic heterogeneity exists and (iii) use the DNA sequence information to establish a formal description of the expected restriction patterns for each genomovar.

Materials and methods

Bacteria and culture conditions

To develop a formal description of the expected restriction patterns for each of the described genomovars, we wanted to ensure that the isolates selected for this study represented each of the different genomovars described to date. The majority of the *F. columnare* isolates in our inventory were retyped using the RFLP methods described in this study. Seven isolates of *F. columnare* were included in this study and were isolated from

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various geographical regions and fish species, and all but one had been previously typed by RFLP (Table 1). These isolates were representative of the diversity of restriction patterns observed. *Flavobacterium columnare* 90–106, a known genomovar III isolate (Darwish & Ismaiel 2005), was included in RFLP analyses to assist in genomovar assignment. The isolates were confirmed as *F. columnare* by PCR as described by Welker *et al.* (2005). Archived glycerol stocks of each isolate were used to inoculate 25 mL of modified Shieh broth (LaFrentz & Klesius 2009) and then incubated at 28 °C on a shaker set at 175 rpm. Following 24 h of growth, sterile inoculating loops were used to pass each isolate onto modified Shieh agar. Plates were incubated at 28 °C for 48 h, and then single isolated colonies were cultured in 25 mL modified Shieh broth using sterile cotton-tip applicators. The isolates were cultured as described above for 24 h and used for DNA extraction.

PCR amplification of 16S rRNA gene

Genomic DNA was extracted from each isolate using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol for Gram-negative bacteria, and the total DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop). A portion of the 16S rRNA gene was amplified from each *F. columnare* isolate by PCR using the 20F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1500R (5′-CGATCCTACTTGCGTAG-3′) primers (Weisburg *et al.* 1991; Triyanto & Wakabayashi 1999). PCR was performed using the HotStarTaq Plus Master Mix Kit (Qiagen), and the final concentrations of each component in the 50 μL reaction mixtures were as follows: 1× HotStarTaq Plus Master Mix, 0.4 μM each primer, 1× CoralLoad Concentrate and 100 ng genomic DNA. PCR amplification was performed with a Primus HTD thermocycler (MWG AG Biotech), and the following cycling protocol was used: 1 cycle of 5 min at 95 °C; 40 cycles of 30 s at 94 °C, 45 s at 55 °C and 60 s at 72 °C; and a final cycle of 10 min at 72 °C. The PCR amplicons were detected by subjecting 3 μL of the PCR products to 0.8% (w/v) agarose (molecular biology grade; Bio-Rad) gel electrophoresis in Tris–acetate–EDTA (TAE) buffer. The gels were precast with 1× SYBR Safe DNA gel stain (Invitrogen), and the PCR products were visualized using ultraviolet transillumination.

RFLP analysis

Restriction fragment length polymorphism analysis was performed by digesting PCR products from each isolate with *Hae*III restriction endonuclease. Fifty microlitres of restriction reaction mixture was prepared by mixing 44 μL PCR product with 1 μL *Hae*III (10 units; New England BioLabs) and 5 μL 10× NEBuffer 4. Restriction reaction mixture was incubated at 37 °C for 1 h, and then two electrophoresis conditions were tested to optimize the resolution of digested PCR products. In the first, digested PCR products were subjected to agarose gel electrophoresis with 2% (w/v) molecular biology–grade agarose (Bio-Rad) in TAE buffer. In the second, the digested PCR products were subjected to agarose gel electrophoresis with 3% (w/v) 3:1 HRB agarose (Amresco) in Tris–borate–EDTA (TBE) buffer. Under both tested

Table 1 Description of *Flavobacterium columnare* isolates used in this study, including the year and fish host of isolation, geographical origin and previous genomovar assignment

Isolate	Year of isolation	Fish host	Origin	Genomovar
ATCC 23463[T]	1955	Chinook salmon *Oncorhynchus tshawytscha* (Walbaum)	Washington (USA)	I[a]
90–106	1990	Channel catfish	Mississippi (USA)	III[b]
ARS-1	1996	Channel catfish	Alabama (USA)	I[a]
ALG-00-530	2000	Channel catfish	Alabama (USA)	II[a]
PT-14-00-151	2000	Channel catfish	Mississippi (USA)	II-B[c]
GA-02-14	2002	Rainbow trout	Georgia (USA)	I-B[c]
F10-HK-A	2012	Yellow perch *Perca flavescens* (Mitchill)	Indiana (USA)	NPD

NPD, not previously determined.
[a]Arias *et al.* (2004).
[b]Darwish & Ismaiel (2005).
[c]Olivares-Fuster *et al.* (2007b).
[T]Type strain.

conditions, the entire (50 μL) restriction reaction mixture was loaded into gels precast with 1× SYBR Safe DNA gel stain and gels were ran at 2.5 V cm^{-1} for 3 h. Following electrophoresis, DNA fragments were visualized using ultraviolet transillumination.

Cloning and sequencing of partial 16S rRNA genes

To detect potential intragenomic heterogeneity, amplified 16S rRNA genes were cloned and sequenced from each *F. columnare* isolate, with the exception of isolate 90–106. The 16S rRNA gene was amplified from each *F. columnare* isolate by PCR using the typing primers 20F and 1500R. PCR was performed with the Accuprime *Pfx* DNA polymerase (Invitrogen), and the final concentrations of each component in the 50 μL reaction mixture were as follows: 1× reaction mix, 0.4 μM each primer, 1 unit polymerase and 120 ng total DNA. PCR amplification was performed with a Primus HTD thermocycler (MWG AG Biotech), and the following cycling protocol was used: 1 cycle of 5 min at 95 °C; 25 cycles of 30 s at 95 °C, 45 s at 55 °C and 78 s at 68 °C; and a final cycle of 10 min at 68 °C. Following amplification, PCR products were purified and concentrated two times using the QIAquick PCR Purification Kit (Qiagen) followed by cloning with the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) according to the manufacturer's protocol. Briefly, PCR products were cloned into pCR®4Blunt-TOPO vector, transformed into One Shot® TOP10 and then spread-plated onto Luria–Bertani (LB) agar plates containing 50 μg mL^{-1} kanamycin. Following overnight growth at 37 °C, 32 individual colonies per isolate were cultured in LB broth containing 50 μg mL^{-1} kanamycin and incubated at 37 °C with shaking at 225 rpm for approximately 16 h. Plasmid DNA was extracted from each transformant using the QIAprep® Miniprep Kit (Qiagen), and isolated plasmids were analysed for the correct insert by restriction analysis with *Eco*RI-HF (New England BioLabs). For each *F. columnare* isolate, insert DNA from 16 to 32 plasmids was sequenced in both directions using vector-specific primers M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers. Sequencing was performed using ABI Big Dye® Terminator v3.1 chemistry on an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). Sequence reads obtained from each plasmid were assembled using BioNumerics version 6.6 (Applied Maths), and sequences were verified by manually examining chromatograms. Vector sequences were trimmed, and then each insert 16S rRNA gene sequence was digested *in silico* with *Hae*III using NEBcutter v2.0 (Vincze, Posfai & Roberts 2003) to determine theoretical restriction patterns. The 16S rRNA gene sequences obtained from each isolate were aligned; a consensus sequence was generated and then examined for single nucleotide polymorphisms (SNPs). Differing sequences obtained from each isolate were deposited in GenBank under accession numbers KC912647–KC912684.

Verification of *Hae*III RFLP patterns

To verify the presence of *Hae*III restriction site polymorphisms that were detected by sequencing, a further experiment was performed. Cloned plasmids containing insert 16S rRNA gene sequences that were representative of each of the identified *Hae*III RFLP patterns were typed by RFLP. The insert DNA was amplified from extracted plasmids by PCR using the vector-specific primers T3 (5′-ATTAACCCTCACTAAAGGGA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′). The vector-specific PCR primers were used to avoid the possibility of amplifying the 16S rRNA gene of *E. coli* with the universal 20F/1500R primers from genomic DNA that may be present in the plasmid extracts. PCR was performed using the HotStarTaq Plus Master Mix Kit (Qiagen), and the final concentrations of each component in the 40 μL reaction mixture were as follows: 1× HotStarTaq Plus Master Mix, 0.4 μM each primer, 1× CoralLoad Concentrate and 60 ng plasmid DNA. PCR amplification was performed with a Primus HTD thermocycler (MWG AG Biotech), and the following cycling protocol was used: 1 cycle of 5 min at 95 °C; 25 cycles of 30 s at 94 °C, 45 s at 50 °C and 60 s at 72 °C; and a final cycle of 10 min at 72 °C. Following gel electrophoresis of PCR products, RFLP analysis was performed by double-digesting PCR products with *Eco*RI-HF and *Hae*III restriction endonucleases. Digestion with *Eco*RI-HF was performed to remove the majority of vector DNA added to the PCR products following amplification with the vector-specific primers, and no *Eco*RI sites were

present in the insert DNA. Forty microlitres of restriction reaction mixture was prepared by mixing 34 μL PCR product with 0.25 μL *Eco*RI-HF (5 units; New England BioLabs), 1 μL *Hae*III (10 units; New England BioLabs), 4 μL 10× NEBuffer 4 and 0.75 μL deionized water. Restriction reaction mixture was incubated at 37 °C for 1 h, and then digested PCR products were subjected to agarose gel electrophoresis using 3:1 HRB agarose (Amresco) and TBE buffer as described above.

Results

Electrophoresis conditions for RFLP

PCR amplification of the 16S rRNA gene from each isolate with the 20F and 1500R primers resulted in a PCR product with an approximate molecular weight of 1300 bp (Fig. 1). The PCR products were then digested with *Hae*III and subjected to two electrophoresis conditions. The DNA fragments were better resolved in a gel composed of 3% 3:1 HRB agarose in TBE buffer (Fig. 2). For each isolate, more DNA fragments were observed when resolved under this condition compared with the same digests ran in 2% molecular biology–grade agarose in TAE buffer (Fig. 2). The cumulative molecular weight of all DNA fragments observed for the ATCC 23463^T and ALG-00-530 isolates was approximately that of the undigested PCR products (i.e. 1300 bp), while those of the other isolates were >1300 bp.

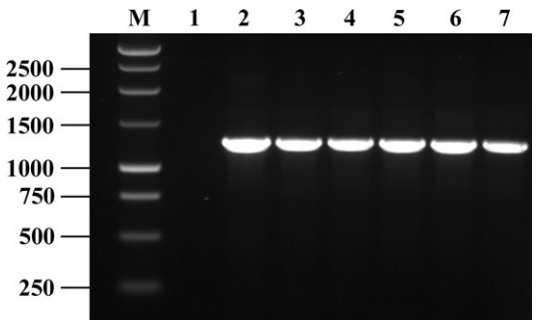

Figure 1 Agarose gel electrophoresis of partial 16S rRNA gene PCR products amplified from *Flavobacterium columnare* isolates using the typing primers (20F and 1500R). Lane M: 1-kb DNA ladder; Lane 1: no template control; Lane 2: ATCC 23463^T; Lane 3: ALG-00-530; Lane 4: GA-02-14; Lane 5: ARS-1; Lane 6: PT-14-00-151; Lane 7: F10-HK-A. Molecular weight markers (bp) are indicated to the left of the gel.

Identification and verification of *Hae*III RFLP patterns

All sequences obtained from each *F. columnare* isolate were 1254 bp in length, which was consistent with the size of the undigested PCR products following electrophoresis (Fig. 1). Examination of all sequences revealed five *Hae*III restriction sites located at positions 181, 264, 616, 907 and 1183 bp (Fig. 3). The restriction sites at positions 616 and 907 were present in every sequence; however, in some of the sequences, one or two of the

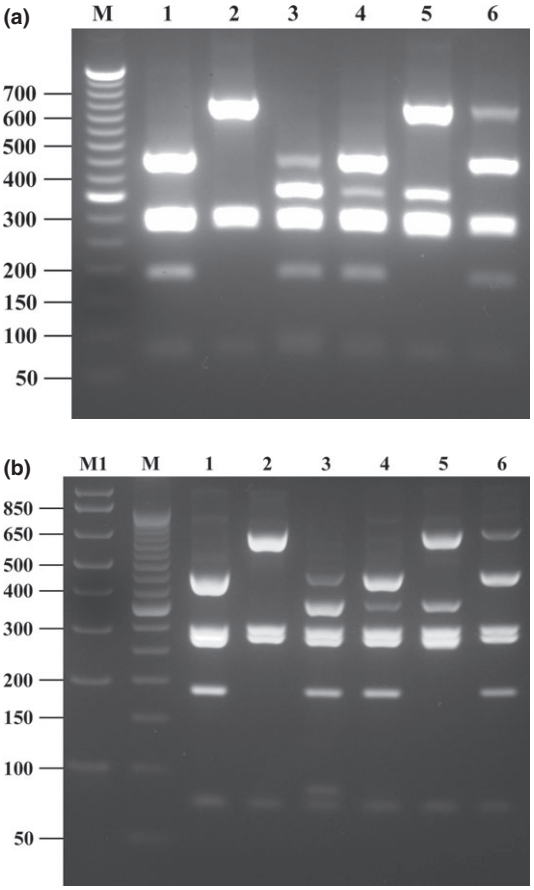

Figure 2 Restriction patterns of partial 16S rRNA gene products amplified from *Flavobacterium columnare* isolates and digested with *Hae*III. Two electrophoresis conditions were compared: (a) 2% molecular biology–grade agarose in Tris–acetate–EDTA (TAE) and (b) 3% 3:1 HRB agarose in Tris–borate–EDTA (TBE). Lane M1: 1-kb DNA ladder; Lane M: 50-bp DNA ladder; Lane 1: ATCC 23463^T (genomovar I); Lane 2: ALG-00-530 (genomovar II); Lane 3: GA-02-14 (genomovar III); Lane 4: ARS-1 (genomovar III); Lane 5: PT-14-00-151 (genomovar II-B); Lane 6: F10-HK-A (genomovar I/II). Molecular weight markers (bp) are indicated to the left of the gels.

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*Hae*III restriction sites were abolished due to SNPs, resulting in fusion of *Hae*III fragments and thus generation of different *Hae*III RFLP patterns. Five *Hae*III RFLP patterns were identified (Fig. 3). To verify the SNPs in the DNA sequences resulting in these patterns, five cloned plasmids containing insert 16S rRNA gene sequences representative of the identified *Hae*III RFLP patterns were typed by RFLP. In addition to *Hae*III digestion, PCR products were also digested with *Eco*RI-HF to remove vector DNA. Complete removal of vector DNA was not possible, resulting in the PCR products containing seven additional bp from the vector at position 0 and 11 bp at position 1254 of the 16S rRNA gene sequences. The observed restriction patterns were consistent with the theoretical *Hae*III RFLP patterns (Figs 3 & 4), with the exception of the *Hae*III RFLP patterns 3 and 5. For *Hae*III RFLP pattern 3, the 71-bp DNA fragment was not observed (Fig. 4), because this fragment contains the 11 bp of additional vector DNA. Thus, the size of this fragment was 82 bp, which is about the same size as the expected 83-bp fragment for this *Hae*III RFLP pattern. The presence of these two similarly sized DNA fragments was reflected by increased band intensity at this molecular weight. For *Hae*III RFLP pattern 5, the 264-bp DNA fragment was not observed (Fig. 4), and this was because this fragment contains the 7 bp of additional vector DNA and an inability to resolve DNA fragments with very similar molecular weight.

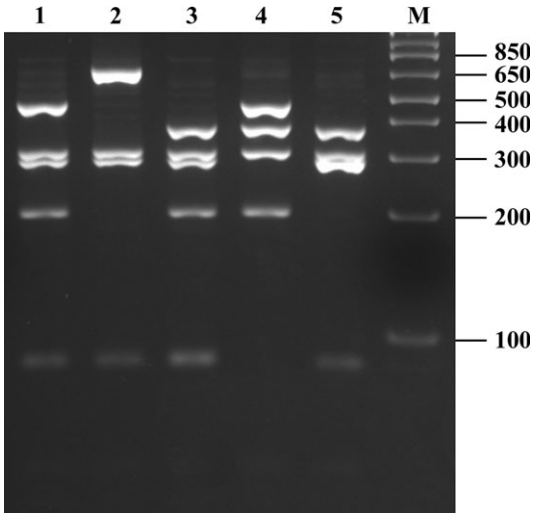

Figure 4 Restriction patterns of partial *Flavobacterium columnare* 16S rRNA gene sequences amplified from cloned plasmid DNA representing each of the five identified *Hae*III restriction fragment length polymorphism (RFLP) patterns. Following PCR amplification of insert DNA using vector-specific primers, products were double-digested with *Hae*III and *Eco*RI-HF. Lane 1: *Hae*III RFLP pattern 1; Lane 2: *Hae*III RFLP pattern 2; Lane 3: *Hae*III RFLP pattern 3; Lane 4: *Hae*III RFLP pattern 4; Lane 5: *Hae*III RFLP pattern 5; Lane M: 1-kb DNA ladder. Molecular weight markers (bp) are indicated to the right of the gel.

Sequencing and observed restriction patterns

For *F. columnare* isolate ATCC 23463^{T}, 14 16S rRNA gene sequences were obtained and each generated the *Hae*III RFLP pattern 1 (Fig. 3;

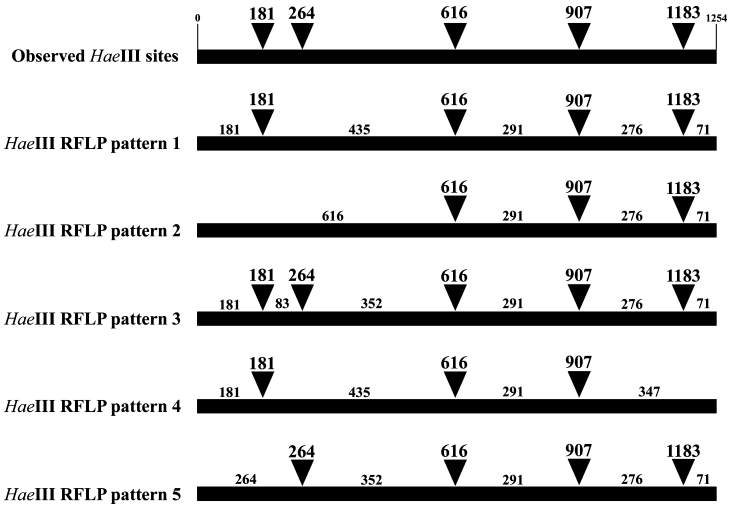

Figure 3 Graphical representation of the *Hae*III restriction sites present in the five *Hae*III restriction fragment length polymorphism (RFLP) patterns identified among the 16S rRNA gene sequences obtained from the six *Flavobacterium columnare* isolates. Closed triangles (▼) and the number above them indicate the location of *Hae*III restriction sites. The numbers between the *Hae*III restriction sites indicate the molecular weight (bp) of the DNA fragments generated following digestion.

Table 2). This theoretical restriction pattern was consistent with the observed pattern following agarose electrophoresis (Fig. 2). Alignment of the 14 sequences revealed seven nucleotide positions with SNPs (Fig. S1).

Eight 16S rRNA gene sequences were obtained for *F. columnare* isolate ALG-00-530 and each generated the *Hae*III RFLP pattern 2 (Fig. 3; Table 2). This theoretical pattern was consistent with the observed restriction pattern following agarose electrophoresis (Fig. 2). Two nucleotide positions exhibited SNPs among the sequences (Fig. S1).

Analysis of the 16S rRNA gene sequences obtained from isolate GA-02-14 suggested the presence of intragenomic heterogeneity. Fourteen of the sequences generated the *Hae*III RFLP pattern 3 (Fig. 3; Table 2). The other two sequences exhibited an SNP at position 266, which abolished the *Hae*III restriction site at position 264, resulting in the *Hae*III RFLP pattern 1 (Fig. 3; Table 2). Thus, the presence of two 16S rRNA genes generating these *Hae*III RFLP patterns in the genome of isolate GA-02-14 would result in a theoretical restriction pattern comprised of DNA fragments of 435, 352, 291, 276, 181, 83 and 71 bp, and the observed restriction pattern was consistent with this (Fig. 2). In addition to the SNP at position 266, eight other positions with SNPs were identified that did not alter *Hae*III sites (Fig. S1).

Analysis of the 16S rRNA gene sequences obtained from the isolate ARS-1 suggests that within the genome of this isolate, three distinct copies of the 16S rRNA gene generate different *Hae*III RFLP patterns due to intragenomic heterogeneity. Four of the sequences generated *Hae*III RFLP pattern 3 (Fig. 3; Table 2). Ten sequences exhibited an SNP at position 266, which abolished the *Hae*III restriction site at position 264, resulting in the *Hae*III RFLP pattern 1 (Fig. 3; Table 2). One sequence exhibited SNPs at positions 266 and 1182, which abolished the *Hae*III restriction sites at positions 264 and 1183, resulting in the *Hae*III RFLP pattern 4 (Fig. 3; Table 2). Thus, the presence of three different 16S rRNA genes generating these *Hae*III RFLP patterns in the genome of isolate ARS-1 would result in a theoretical restriction pattern comprised of DNA fragments of 435, 352, 347, 291, 276, 181, 83 and 71 bp. The 347-bp DNA fragment was not visible in the observed restriction pattern due to the presence of the 352-bp band and the inability to resolve these DNA fragments with very similar molecular weight (Fig. 2). The 83-bp DNA fragment was faint (Fig. 2), but could be observed following manipulation of the gel image. In addition to the SNPs at nucleotide positions 266 and 1182, eight other positions with SNPs were identified that did not alter *Hae*III sites (Fig. S1).

Analysis of the 16S rRNA gene sequences obtained from isolate PT-14-00-151 suggested the presence of intragenomic heterogeneity. Six of the sequences generated the *Hae*III RFLP pattern 5 (Fig. 3; Table 2). The remaining five sequences exhibited an SNP at position 266, which abolished the *Hae*III restriction site at position 264, resulting in the *Hae*III RFLP pattern 2 (Fig. 3; Table 2). Thus, the presence of two different 16S rRNA genes generating these *Hae*III RFLP patterns in the genome of this isolate would result in

Table 2 The number of plasmids containing insert DNA sequenced for each *Flavobacterium columnare* isolate and the restriction pattern obtained following *in silico* digestion of the partial 16S rRNA gene sequences with *Hae*III. Each unique restriction pattern identified was assigned an *Hae*III RFLP pattern number (1–5)

Isolate	Number of plasmids	*In silico* restriction pattern (bp)									*Hae*III RFLP pattern
ATCC 23463^T	14		435			291	276		181		71	1
ALG-00-530	8	616				291	276				71	2
GA-02-14	14			352		291	276		181	83	71	3
	2		435			291	276		181		71	1
ARS-1	10		435			291	276		181		71	1
	1		435		347	291			181			4
	4			352		291	276		181	83	71	3
PT-14-00-151	5	616				291	276				71	2
	6			352		291	276	264			71	5
F10-HK-A	27		435			291	276		181		71	1
	5	616				291	276				71	2

RFLP, restriction fragment length polymorphism.
^{T}Type strain.

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a theoretical restriction pattern comprised of DNA fragments of 616, 352, 291, 276, 264 and 71 bp. The DNA fragment of 264 bp was not visible in the observed restriction pattern (Fig. 2). In addition to the SNP at nucleotide position 266, five other positions with SNPs were identified that did not alter *Hae*III sites (Fig. S1).

Analysis of the 16S rRNA gene sequences obtained from isolate F10-HK-A suggests that within the genome of this isolate, two copies of the 16S rRNA gene generate different *Hae*III RFLP patterns due to intragenomic heterogeneity. Twenty-seven of the sequences generated the *Hae*III pattern 1 (Fig. 3; Table 2). The remaining five sequences exhibited an SNP at position 183, which abolished the *Hae*III restriction site at position 181, resulting in the *Hae*III RFLP pattern 2 (Fig. 3; Table 2). Thus, the presence of two different 16S rRNA genes generating these *Hae*III RFLP types in the genome of this isolate would result in a theoretical restriction pattern comprised of DNA fragments of 616, 435, 291, 276, 181 and 71 bp, which was consistent with the observed pattern (Fig. 2). In addition to the SNP at nucleotide position 183, five other positions with SNPs were identified that did not alter *Hae*III sites (Fig. S1).

RFLP of GA-02-14, ARS-1 and 90–106

To assist in genomovar assignment, a known genomovar III *F. columnare* isolate, 90–106, was obtained. The 90–106, GA-02-14 and ARS-1 isolates were typed by RFLP to compare observed restriction patterns. Each isolate exhibited the same number and sizes of DNA fragments; however, the staining intensities of the 435-, 352- and 83-bp fragments were variable (Fig. 5).

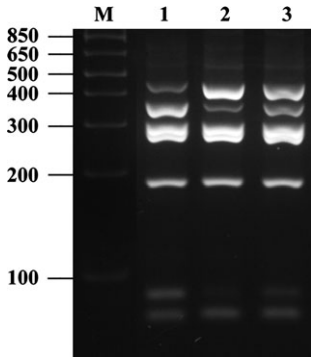

Figure 5 Restriction patterns of partial 16S rRNA gene products amplified from genomovar III isolates of *Flavobacterium columnare* and digested with *Hae*III. Lane M: 1-kb DNA ladder; Lane 1: GA-02-14; Lane 2: ARS-1; Lane 3: 90–106. Molecular weight markers (bp) are indicated to the left of the gel.

Discussion

For the initial efforts to type the *F. columnare* isolates in our inventory, TAE and general laboratory-grade agarose were used for electrophoresis. Following a comparison of the observed restriction patterns with those published by Darwish & Ismaiel (2005), it was evident that improved electrophoresis conditions were needed to increase the resolution of DNA fragments. The use of TBE as the electrophoresis buffer and 3:1 HRB agarose consistently resulted in increased resolution as evidenced by the visualization of additional DNA fragments that were not observed when TAE and laboratory-grade agarose were used (Fig. 2). The increased resolution made it possible to effectively compare predicted restriction patterns obtained from DNA sequences with observed restriction patterns obtained following electrophoresis.

The genome of *Flavobacterium columnare* AT CC 49512, a genomovar I isolate, has been sequenced, annotated and published (Tekedar *et al.* 2012). Multiple copies of the 16S rRNA gene are common among bacterial genomes that have been sequenced (Pei *et al.* 2010), and similarly, the ATCC 49512 genome contains five copies of the 16S rRNA gene. Each copy is identical in nucleotide sequence (100% identity), and when the partial gene sequences are digested *in silico* with *Hae*III, each generates a theoretical restriction pattern consistent with a genomovar I isolate. In contrast to the *F. columnare* ATCC 49512 genome, our sequences suggested the presence of intragenomic heterogeneity among copies of the 16S rRNA genes within the genome of each *F. columnare* isolate. Intragenomic heterogeneity in the 16S rRNA genes has been observed in other bacterial species (Moreno, Romero & Espejo 2002; Coenye & Vandamme 2003; Acinas *et al.* 2004; Alperi *et al.* 2008; Arias, Olivares-Fuster & Goris 2010). In a few studies, the observed heterogeneity occurred within restriction sites that were used for typing the bacterial species, resulting in atypical restriction patterns (Alperi *et al.* 2008; Arias *et al.* 2010; Figueras, Levican & Collado 2012). Similarly, for some of the *F. columnare* isolates in

the present study, intragenomic heterogeneity within *Hae*III restriction sites resulted in at least two copies of the 16S rRNA genes exhibiting sequences that generate different *Hae*III RFLP patterns. This finding explains why the cumulative molecular weight of all DNA fragments observed for these isolates was greater than that of the undigested PCR product.

The expected restriction patterns for genomovar I and II isolates (Table 3), derived from the *Flavobacterium columnare* ATCC 23463^T and ALG-00-530 isolates, are consistent with the original 16S rRNA gene sequences deposited by Triyanto & Wakabayashi (1999). The original genomovar I sequences (accession nos. AB010952, AB010951 and AB023660) and one of the original genomovar II DNA sequences (isolate LP 8, accession no. AB015480) deposited by Triyanto & Wakabayashi (1999) generate these same restriction patterns when digested *in silico* with *Hae*III (data not shown). *In silico* digestion of the other genomovar II DNA sequence (isolate EK 28; accession no. AB016515) deposited by these authors does not match this pattern (data not shown) or any of the restriction patterns identified in this study. However, the observed restriction pattern of isolate EK 28 was identical to isolate LP 8 (Triyanto & Wakabayashi 1999), suggesting possible errors in the DNA sequence obtained for isolate EK 28.

In the present study, the observed restriction pattern for GA-02-14, previously typed as genomovar I-B (Olivares-Fuster *et al.* 2007b), was similar to the patterns obtained by these authors for this same isolate and another genomovar I-B isolate; however, after critical comparison with the patterns published by Triyanto & Wakabayashi (1999) and Darwish & Ismaiel (2005), the restriction pattern was nearly identical to the patterns obtained with genomovar III isolates. Therefore, a known genomovar III isolate, 90–106 (Darwish & Ismaiel 2005), was acquired and typed, and the generated restriction pattern exhibited the same number and sizes of DNA fragments as the GA-02-14 isolate (Fig. 5; Table 3). This demonstrates that the isolates previously described as genomovar I-B are not a subtype of genomovar I, but rather genomovar III. This is further supported by the DNA sequences that were deposited by Schneck & Caslake (2006), in which the authors amplified the 16S rRNA gene from two genomovar III *F. columnare* isolates (Catfish-94 and Baitfish-91), then cloned and sequenced the PCR products. *In silico* digestion of the deposited sequence from the Catfish-94 isolate (accession no. DQ005509) generates the *Hae*III RFLP pattern 1, and the other deposited sequence from the Baitfish-91 isolate (accession no. DQ005511) generates the *Hae*III RFLP pattern 3. The original genomovar III sequence from isolate PH 97028 (accession no. AB015481) deposited by Triyanto & Wakabayashi (1999) also generates the *Hae*III RFLP pattern 3. These findings support our sequencing results.

The observed restriction pattern for *F. columnare* ARS-1, previously typed as genomovar I (Arias *et al.* 2004), exhibited the same number and sizes of DNA fragments as a genomovar III isolate and therefore was assigned to this genetic type (Table 3). It should be noted that this particular isolate also harbours a 16S rRNA gene whose sequence generates the *Hae*III RFLP pattern 4. However, the bands generated from this pattern would not likely be observed following agarose electrophoresis because they are identical in size to bands generated from the *Hae*III RFLP patterns 1 and 3, with the exception of the 347-bp band, which is of similar size as the 352-bp band from the *Hae*III RFLP pattern 3 (Fig. 3).

The observed restriction pattern for *F. columnare* PT-14-00-151, previously typed as a genomovar II-B (Olivares-Fuster *et al.* 2007b), can be

Table 3 Expected restriction patterns of *Flavobacterium columnare* genomovars, based upon partial 16S rRNA gene sequences and *in silico* digestion with *Hae*III

Genomovar	Restriction pattern (bp)									Isolate
I		435		291	276		181		71	ATCC 23463^T
II	616			291	276				71	ALG-00-530
II-B	616		352	291	276	264[a]			71	PT-14-00-151
III		435	352	291	276		181	83[a]	71	GA-02-14; ARS-1
I/II	616	435		291	276		181		71	F10-HK-A

[a]The identified DNA fragment may be faint or not observed following agarose electrophoresis.
^{T}Type strain.

differentiated from the observed genomovar II pattern by the presence of a 352-bp band and possibly a 264-bp band (Fig. 2; Table 3). Therefore, the isolates with this restriction pattern are formally described as genomovar II-B (Table 3).

To our knowledge, the observed restriction pattern for *F. columnare* F10-HK-A has not been previously described. As the observed restriction pattern appears as a hybrid between a genomovar I and II isolates, we propose that isolates exhibiting this expected restriction pattern be assigned to genomovar I/II (Table 3).

The majority of the *F. columnare* isolates in our archived inventory were typed as genomovar I, II or III, and the other genetic types were relatively uncommon. However, the existence of other isolates that exhibit different observed restriction patterns is possible for a number of reasons. Five *Hae*III RFLP patterns were identified in the present work (Figs 3 & 4), and it is possible that any combination of these may be present in a given *F. columnare* isolate. Secondly, other *Hae*III RFLP patterns are possible if additional polymorphisms occur within any of the identified *Hae*III restriction sites. Thirdly, within the portion of the 16S rRNA gene used for typing, 25 other *Hae*III restriction sites are possible should an SNP occur at the correct position with the proper nucleotide (Fig. S1). This may be likely based on the number of locations observed with SNPs in each isolate. Each isolate exhibited at least two locations with SNPs among the partial 16S rRNA gene inserts that were sequenced, and the greatest number of locations was 10. Because only the SNPs within the *Hae*III restriction sites were confirmed, the number of other locations with SNPs may be an overestimate as some of these may be artefacts that can occur when PCR-amplifying and cloning very closely related gene products (Speksnijder *et al.* 2001).

One final factor that may contribute to different observed restriction patterns is the number of 16S rRNA genes encoded in an isolate's genome whose sequence generates a different *Hae*III RFLP pattern. Although the present work demonstrated the existence of up to three 16S rRNA genes in a single *F. columnare* genome, it is not known how many copies of the 16S rRNA genes were attributed to each of these *Hae*III RFLP patterns. Therefore, following electrophoresis of the digested PCR products, the staining intensities of DNA fragments may be different than observed in the present study if an isolate harbours a different number of 16S rRNA genes corresponding to the different *Hae*III RFLP patterns. This is exemplified by the three genomovar III isolates typed in the present study. The observed restriction patterns are identical in terms of the number and sizes of DNA fragments; however, the staining intensity of the 435-, 352- and 83-bp fragments differs (Fig. 5). The restriction pattern of the ARS-1 isolate differs from that of the GA-02-14 isolate by a more intensely stained 435-bp band and less intensely stained 352- and 83-bp bands (Fig. 5). These results suggest that the ARS-1 isolate harbours more copies of the 16S rRNA gene sequence generating the *Hae*III RFLP pattern 1 (producing the 435-bp fragment) and fewer copies of the gene sequence generating the *Hae*III RFLP pattern 3 (producing the 352- and 83-bp fragments) as evidenced by the number of plasmids sequenced from these isolates that exhibited these RFLP patterns (Table 2). Although no sequencing was performed, the observed restriction pattern of isolate 90–106 suggests that the copy number of the 16S rRNA gene sequences generating the *Hae*III RFLP patterns 1 and 3 may be similar because the 435-bp and 352-bp bands are of similar staining intensity.

It has been suggested that genomovar I *F. columnare* isolates are less virulent in three fish species than genomovar II isolates (Shoemaker *et al.* 2008; Olivares-Fuster *et al.* 2011; LaFrentz *et al.* 2012). The results of the present study suggest that the association between virulence and genomovar should be re-examined because some of the isolates used in those studies have been reassigned to different genomovars. For instance, Shoemaker *et al.* (2008) determined the virulence of three genomovar I and four genomovar II isolates in channel catfish fry. Two of the genomovar I isolates used, ARS-1 and HS, have now been correctly assigned to genomovar III (data not shown for isolate HS). The ARS-1 isolate was also used in the studies of Olivares-Fuster *et al.* (2011) and LaFrentz *et al.* (2012). Shoemaker *et al.* (2008) also determined the virulence of three genomovar I and three genomovar II isolates in channel catfish fingerlings. Two of the genomovar I isolates used, ALM-05-53 and ALM-05-140, have now been correctly assigned to genomovar III. These two isolates and the ARS-1 isolate are of particular interest because they were virulent (mean cumulative mortality of approximately 45%). This finding indicates that genomovar III

isolates are virulent in channel catfish. Upon examination of the literature, this has been demonstrated previously. Soto *et al.* (2008) tested the virulence of *F. columnare* 90–106 in channel catfish, which is a genomovar III isolate (Darwish & Ismaiel 2005), and approximately 65% mortality was observed. Thomas-Jinu & Goodwin (2004) reported high and moderate mortality in channel catfish and golden shiners *Notemigonus crysoleucas* (Mitchill), respectively, following challenge with *F. columnare* isolate AL-94-203. This isolate was subsequently typed as genomovar III by Schneck & Caslake (2006). However, genomovar III isolates have not been studied in detail and therefore warrant further investigation.

LaFrentz *et al.* (2012) suggested a host-specific association between salmonids and genomovar I isolates due to the observation that all published *F. columnare* isolates typed from salmonids were genomovar I. However, with the new knowledge gained in the present research, the GA-02-14 isolate that was obtained from a rainbow trout in Georgia (USA) has now been correctly assigned to genomovar III. To our knowledge, this is the first report of the isolation of a genomovar other than genomovar I from a salmonid. It has been established that genomovars I, II and III are present in the south-eastern USA (Darwish & Ismaiel 2005; Olivares-Fuster *et al.* 2007a); therefore, it is conceivable that a genomovar III isolate could be recovered from a salmonid reared in this region. However, it is not currently known whether genetic types of *F. columnare*, other than genomovar I, are present in the Hagerman Valley of Idaho, where the majority of rainbow trout production occurs in the USA. This region is unique due to the presence of spring-fed 15 °C water and geothermal water. The availability of these water sources has led to diverse aquaculture production consisting of rainbow trout as well as some warmwater fish species such as tilapia *Oreochromis* sp., channel catfish and ornamental fish. The importation of warmwater fish species originating from outside of this region may pose a risk of introducing other genetic types of *F. columnare* and transferring them to rainbow trout that are reared at the same facility. This may negatively impact rainbow trout production because genomovar II isolates were shown to be highly virulent in rainbow trout (LaFrentz *et al.* 2012), and the reassigning of the GA-02-14 isolate to genomovar III indicates that these isolates can infect this fish species. Further support for the potential virulence of genomovar III isolates in rainbow trout was provided by LaFrentz *et al.* (2012), in which the ARS-1 isolate (now correctly assigned to genomovar III) was used in a challenge experiment and resulted in mean mortality of 23%. The standard protocol for the RFLP method described in this study may serve as a useful tool for determining the presence of *F. columnare* genomovars in aquaculture facilities, source water or geographical regions, such as the Hagerman Valley of Idaho.

In summary, this research provides a standard protocol for typing isolates of *F. columnare* by RFLP and a formal description of the expected restriction patterns for the previously described genomovars I, II, II-B and III. Additionally, we describe a new genomovar, I/II. With this new knowledge, the analysis of gels and assignment of an unknown *F. columnare* isolate to a genomovar are straightforward, assuming proper controls are included. For this purpose, the authors will provide control DNA from each of the described genomovars in this article upon request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of the partial 16S rRNA consensus gene sequences obtained for each of the six *Flavobacterium columnare* isolates.

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